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VIRUS-CELL INTERACTIONS

Negeviruses Reduce Replication of Alphaviruses during Coinfection

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ABSTRACT Negeviruses are a group of insect-specific viruses (ISVs) that have been found in many arthropods. Their presence in important vector species led us to examine their interactions with arboviruses during coinfections. Wild-type negeviruses reduced the replication of several alphaviruses during coinfections in mosquito cells. Negev virus (NEGV) isolates were also used to express green fluorescent protein (GFP) and anti-chikungunya virus (CHIKV) antibody fragments during coinfections with CHIKV. NEGV expressing anti-CHIKV antibody fragments was able to further reduce replication of CHIKV during coinfections, while reductions of CHIKV with NEGV expressing GFP were similar to titers with wild-type NEGV alone. These results are the first to show that negeviruses induce superinfection exclusion of arboviruses and to demonstrate a novel approach to deliver antiviral antibody fragments with paratransgenic ISVs. The ability to inhibit arbovirus replication and express exogenous proteins in mosquito cells makes negeviruses a promising platform for control of arthropod-borne pathogens.

IMPORTANCE Negeviruses are a group of insect-specific viruses (ISVs), viruses known to infect only insects. They have been discovered over a wide geographical and species range. Their ability to infect mosquito species that transmit dangerous arboviruses makes negeviruses a candidate for a pathogen control platform. Coinfections of mosquito cells with a negevirus and an alphavirus demonstrated that negeviruses can inhibit the replication of alphaviruses. Additionally, modifying Negev virus (NEGV) to express a fragment of an anti-CHIKV antibody further reduced the replication of CHIKV in coinfected cells. This is the first evidence to demonstrate that negeviruses can inhibit the replication of important arboviruses in mosquito cells. The ability of a modified NEGV to drive the expression of antiviral proteins also highlights a method for negeviruses to target specific pathogens and limit the incidence of vector-borne diseases.

KEYWORDS Negev virus, alphavirus, insect-specific virus, negevirus, paratransgenesis, superinfection exclusion

Many insect-specific viruses (ISVs) have been discovered in wild-caught and laboratory colonies of mosquitoes and in mosquito cell cultures (1). ISVs are only known to replicate in arthropods or insect cell lines. While posing no threat to human or animal health, ISVs may affect the transmission of more dangerous vector-borne pathogens. Highly insect-pathogenic ISVs have been suggested for use as biological control

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agents to reduce populations of vector-competent mosquitoes (2–4). Several recent studies have demonstrated that ISVs may play a more direct role by inhibiting the replication of arboviruses within the insect host. The majority of these experiments have attempted to define a relationship based on superinfection exclusion, a phenomenon in which an established virus infection interferes with a secondary infection by a closely related virus. For example, insect-specific flaviviruses, such as cell fusing agent virus (CFAV), Nhumirim virus (NHUV), and Palm Creek virus (PCV), have demonstrated an ability to reduce viral loads of vertebrate pathogenic flaviviruses, like West Nile virus (WNV), Zika virus (ZIKV), dengue virus (DENV), and Japanese (JEV) and St. Louis encephalitis (SLEV) viruses (5–10). Similarly, the insect-specific alphavirus Eilat virus (EILV) was shown to reduce or slow replication of the pathogenic alphaviruses chikungunya virus (CHIKV), Sindbis virus (SINV), and eastern (EEEV), western (WEEV), and Venezuelan equine encephalitis (VEEV) viruses in cell culture or in mosquitoes (11). Less information is available about the effect of unrelated viruses during superinfection. Cell cultures chronically infected with Aedes albopictus densovirus (AalDNV) limit replication of DENV (12), cell cultures with established CFAV and Phasi Charoen-like virus (PCLV) infections reduced ZIKV and DENV replication (13), and coinfections with Yichang virus, a mesonivirus, reduced DENV replication in cells and dissemination in mosquitoes (14). The mechanism for these reduced titers has not been elucidated, but the relationships appear to be virus and even host specific (5, 15, 16).

The genus *Negevirus* is a recently discovered, unclassified group of ISVs (17). Members of this genus have been isolated from several species of hematophagous mosquitoes and sandflies, and negev-like viruses have also been found in other nonvector insects (18–25). Phylogenetic studies have placed this group of viruses most closely to members of the genus *Cilevirus*, plant pathogens that are transmitted by mites (17, 24). These viruses have a single-stranded, positive-sense RNA genome of ~9 to 10 kb and contain three open reading frames (ORFs) (17). The ORFs encode the replication machinery (ORF1), a putative glycoprotein (ORF2), and a putative membrane protein (ORF3). Electron microscopy has shown the structural proteins to be arranged in a hot air balloon morphology, a round particle with a single protrusion that is likely the glycoprotein structure (26–28). Little is known about the infectivity, transmission dynamics, and species range of negeviruses. However, they are commonly found in field-collected mosquitoes (29, 30).

The association of negeviruses with important vector species over a wide geographical range raises the question of possible interactions or interference of negeviruses with vertebrate pathogenic viruses. Few studies exist that demonstrate the ability of unrelated viruses to induce superinfection exclusion, but evidence for this phenomenon with negeviruses could provide a platform to control vector-borne viral diseases in many arthropod vector species. In this study, three negevirus isolates from the Americas were assessed for superinfection exclusion in cell cultures with VEEV, CHIKV Mayaro virus (MAYV), o'nyong-nyong virus (ONNV), and Semliki Forest virus (SFV). The use of a Negev virus (NEGV) infectious clone also allowed manipulation of the virus genome to provide a greater ability to exclude superinfection with CHIKV.

RESULTS

Wild-type negevirus growth curves. All wild-type negeviruses reached titers greater than 10 $\log_{10}$ PFU/ml within 48 h when infected at a multiplicity of infection (MOI) of 1 (Fig. 1A). NEGV and Piura virus (PIUV)-Lutzomyia neared peak titer by 12 h postinfection (hpi), while PIUV-Culex neared peak titer at 24 hpi. Infections of NEGV with MOIs of 1 and 5 produced similar growth curves (Fig. 1B).

Superinfection exclusion of alphaviruses with wild-type negeviruses. To determine the effect of negeviruses on the replication of alphaviruses in cell culture, negevirus isolates were coinfected with VEEV-TC83 or CHIKV isolates. NEGV was able to significantly reduce replication of VEEV-TC83, with reductions of 5.5 to 7.0 $\log_{10}$ PFU/ml of VEEV at 48 h (Fig. 2A). There were no significant differences in VEEV-TC83 titers when coinfected with NEGV inoculated at MOIs of 1 or 5. There were also no significant

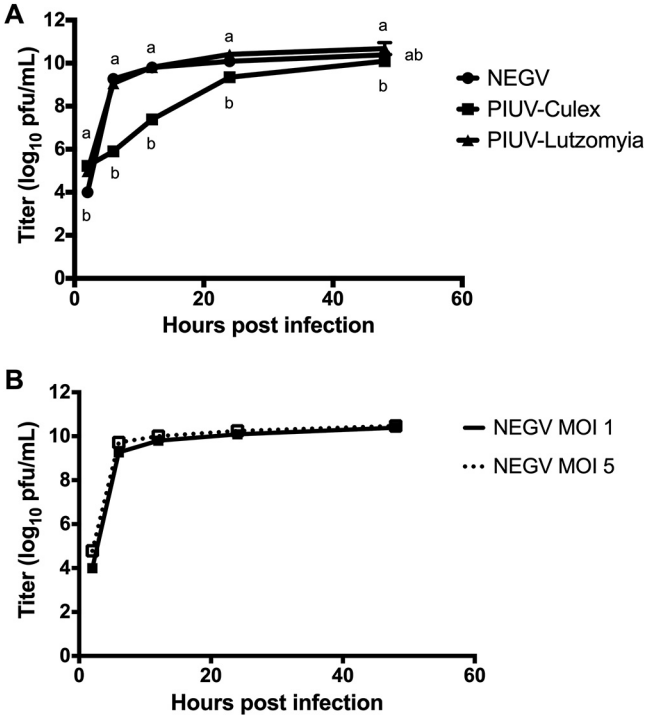

FIG 1 Growth curve for wild-type negeviruses. (A) The titer of each virus, Negev virus (NEGV), Piura virus-Culex (PIUV-Culex), and Piura virus-Lutzomyia (PIUV-Lutzomyia), at different time points following infection at an MOI of 1 in C7/10 cells. (B) Growth curve of NEGV with MOIs of 1 and 5 in C7/10 cells. All points represent means from $n = 3$, ± standard deviations (SD). Letters indicate significant differences ($P < 0.0001$).

differences in VEEV-TC83 titers when NEGV inoculation preceded VEEV-TC83 inoculation by 0, 2, or 6 h. Coinfection with PIUV-Culex or PIUV-Lutzomyia also significantly reduced replication of VEEV-TC83 across all time points (Fig. 2B to D). A similar reduction of VEEV-TC83 was observed during all negevirus coinfections, as VEEV-TC83 was reduced 4.6 to 7.2 log_{10} PFU/ml at 48 h.

Coinfections with CHIKV and NEGV also resulted in significantly lower titers of CHIKV at all time points but only reduced the titer of CHIKV by 0.65 to 0.93 log_{10} PFU/ml after 48 h (Fig. 3A). Varying the MOI of NEGV and timing of CHIKV inoculation only produced differing titers of CHIKV at the 12-h time point. However, titers of CHIKV during coinfection with different negeviruses varied greatly (Fig. 3B and C), with the largest variance of CHIKV titers, reductions of 0.65 log_{10}, 2.4 log_{10}, and 5.3 log_{10} PFU/ml, observed when inoculated 6 h postinoculation with NEGV, PIUV-Culex, and PIUV-Lutzomyia, respectively (Fig. 3D).

NEGV and PIUV-Lutzomyia were also able to reduce replication of several other alphavirus isolates by various amounts when infected simultaneously. Titers of VEEV-IC were reduced by 2.8 to 3.0 log_{10} PFU/ml at 48 h postcoinfection (Fig. 4A). Three isolates of MAYV, Guyane, BeAn343102, and BeAr505411, were reduced by 1.9 to 3.2 log_{10} PFU/ml at 48 h postcoinfection (Fig. 4B). Outputs between MAYV coinfections were similar, with significant differences at 48 h only seen for MAYV-Guyane coinfected with NEGV and PIUV-Lutzomyia, respectively. ONNV titers were reduced by 2.4 to 3.2 log_{10} PFU/ml, and SFV was reduced by 1.2 to 1.8 log_{10} PFU/ml after 48 h (Fig. 4C and D).

Replication of modified NEGV isolates. The sequence for GFP was successfully cloned as both a fusion and cleaved protein at several sites along the NEGV infectious clone (Table 1, Fig. 5A). Following electroporation, viable virus was rescued from isolates with GFP inserted as a fusion and cleaved protein on the C terminus of ORF3 and as a cleaved protein on the C terminus of ORF1.

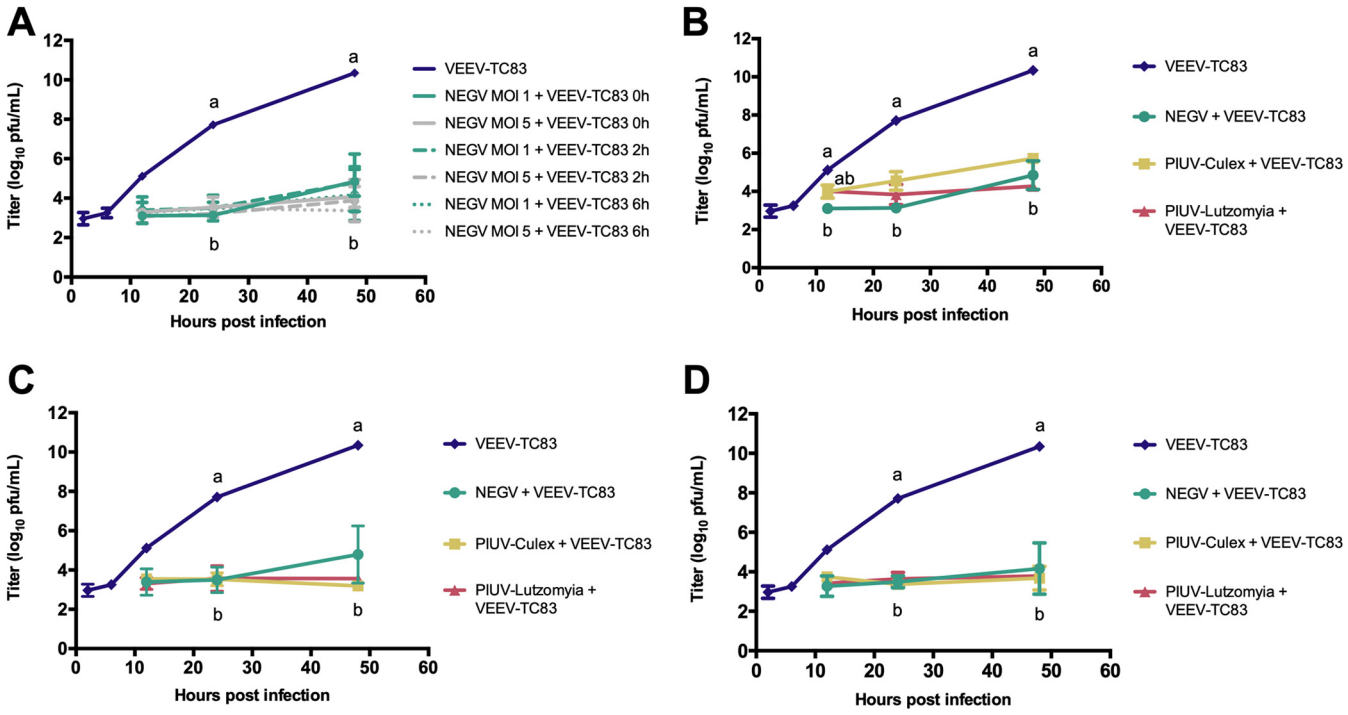

FIG 2 Growth curves of VEEV-TC83 in C7/10 cells during coinfections with wild-type negeviruses. (A) Growth curves of VEEV-TC83 when inoculated on cells at 0, 2, and 6 h after NEGV infections. NEGV was inoculated at an MOI of 1 or 5. (B to D) Growth curves of VEEV-TC83 when inoculated on cells at 0 h after negevirus infection (B), 2 h after negevirus infection (C), and 6 h after negevirus infection (D). Negeviruses were inoculated at an MOI of 1 (B to D). VEEV-TC83 was inoculated at an MOI of 0.1 for all conditions. All points represent means from $n = 3$, ±SD. Letters indicate significant differences ($P < 0.0001$).

Further experiments used isolates with GFP added to ORF3 (NEGV GFP-fusion and NEGV GFP) and also with GFP swapped with single-chain variable fragment (scFv) CHK265, a partial sequence for an anti-CHIKV antibody (Fig. 5A). Mutated isolates were rescued and had titers ranging from 9.6 to 10.4 $\log_{10}$ PFU/ml, with growth curves similar to those of wild-type NEGV (Fig. 5B). Cells infected with NEGV GFP-fusion demonstrated brilliant, punctate fluorescence (Fig. 5C), while cells infected with NEGV GFP (cleaved) demonstrated dull, diffuse fluorescence (Fig. 5D). The number of fluorescent plaques and overall titer of NEGV isolates expressing GFP remained stable for 5 passages in C7/10 cells (Fig. 5E and F).

Superinfection exclusion of alphaviruses with modified NEGV. NEGV isolates expressing GFP or scFv-CHK265 were used to infect cells for coinfection with VEEV-TC83 or CHIKV. The results for coinfections with VEEV-TC83 were similar to the reduction in titer seen with wild-type viruses. At the 48-h time point, VEEV-TC83 was reduced by 4.5 to 5.5 $\log_{10}$ PFU/ml when coinfected with NEGV isolates (Fig. 6A), 4.6 to 5.8 $\log_{10}$ PFU/ml when infected 2 h after NEGV isolates (Fig. 6B), and 5.6 to 6.9 $\log_{10}$ PFU/ml when infected 6 h after NEGV isolates (Fig. 6C). When infected simultaneously with CHIKV, titers were reduced by 0.7 to 1.1 $\log_{10}$ PFU/ml during coinfections of NEGV expressing GFP and by 2.9 to 3.8 $\log_{10}$ PFU/ml during coinfections of NEGV expressing scFv-CHK265 at the 48-h time point (Fig. 7A). When inoculated 2 h after NEGV infection, the titer of CHIKV after 48 h was reduced 0.7 to 0.9 $\log_{10}$ PFU/ml with NEGV expressing GFP and 3.7 to 4.5 $\log_{10}$ PFU/ml with NEGV expressing scFv-CHK265 (Fig. 7B). Delaying CHIKV infection 6 h after NEGV infection resulted in reductions of 1.2 to 1.9 $\log_{10}$ PFU/ml and 5.2 to 5.7 $\log_{10}$ PFU/ml after 48 h of coinfection with NEGV expressing GFP and scFv-CHK265, respectively (Fig. 7C).

DISCUSSION

The microbiome of arthropod vectors is known to influence host-pathogen interactions (31–33). The precise mechanisms of pathogen inhibition are unknown, but there

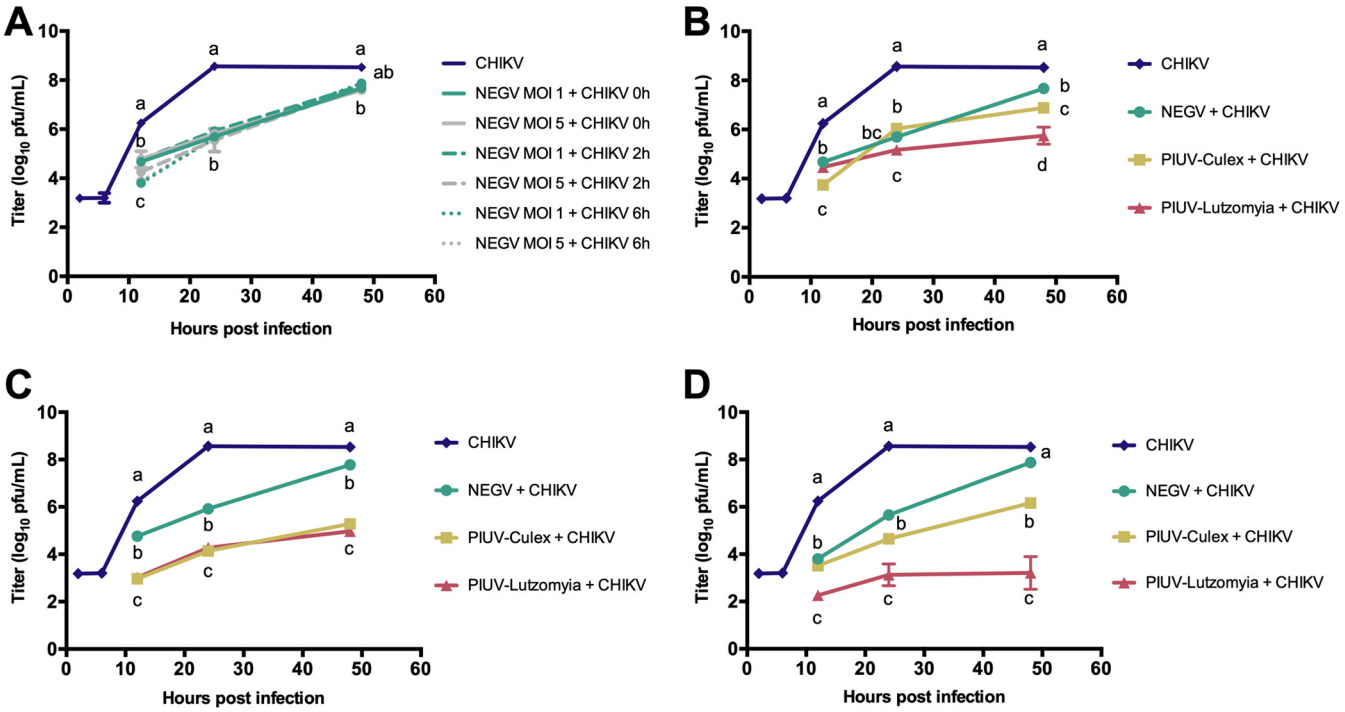

FIG 3 Growth curves of CHIKV in C7/10 cells during coinfections with wild-type negeviruses. (A) Growth curves of CHIKV when inoculated on cells at 0, 2, and 6 h after NEGV infections. NEGV was inoculated at an MOI of 1 or 5. (B to D) Growth curves of CHIKV when inoculated on cells at 0 h after negevirus infection (B), 2 h after negevirus infection (C), and 6 h after negevirus infection (D). Negeviruses were inoculated at an MOI of 1 (B to D). CHIKV was inoculated at an MOI of 0.1 for all conditions. All points represent means from $n = 3$, ±SD. Letters indicate significant differences ($P < 0.0001$).

is increasing evidence that interference from ISVs is one mechanism (5–7, 10). Interactions between related viruses have led to the theory of superinfection exclusion, in which an established infection interferes with or inhibits a secondary infection by a closely related virus. For example, a CFAV mosquito isolate reduced the replication of DENV and ZIKV during coinfections in mosquitoes and mosquito cells (5).

To investigate if superinfection exclusion occurred with other virus combinations, pathogenic alphaviruses and negeviruses were used in coinfection experiments. Titers of multiple VEEV isolates and MAYV isolates were consistently reduced during coinfection experiments with negeviruses. Reductions varied during CHIKV-negevirus coinfections. These results provide further evidence that superinfection exclusion of alphaviruses is pathogen specific but differ from a previous report demonstrating no reduction in titer of VEEV TC-83 and a significant reduction of wild-type VEEV-IC (strain 3908) after 48 h when coinfected with EILV, an alphavirus ISV (11). However, the potential for superinfection exclusion of pathogens is different for each ISV despite their relatedness. These differences have been demonstrated among several insect-specific flaviviruses. NHUV and PCV were capable of superinfection exclusion; CFAV gave various results; and Culex flavivirus (CxFV) did not reduce titers of pathogenic arboviruses (5–10, 15, 16, 34–37). In our experiments with negeviruses, PIUV isolates were more capable than NEGV at inhibiting important arboviruses.

While ISVs show promising results to block arbovirus replication in mosquito vectors, their unknown mechanism of action may limit their use against a wide range of pathogens, but paratransgenic ISVs could be used to provide antiviral molecules that specifically interfere with pathogen transmission (38). To this end, we used an infectious clone of NEGV to deliver a fragment of an antibody known to neutralize CHIKV (39). An scFv consists of the variable regions of the heavy and light chains of an antibody, joined by a soluble linker. These antibody fragments can possess the neutralizing qualities of their full-size versions in only ~27 kDa. Coinfections with scFv-expressing NEGV isolates greatly reduced titers of CHIKV, whereas coinfections with control NEGV

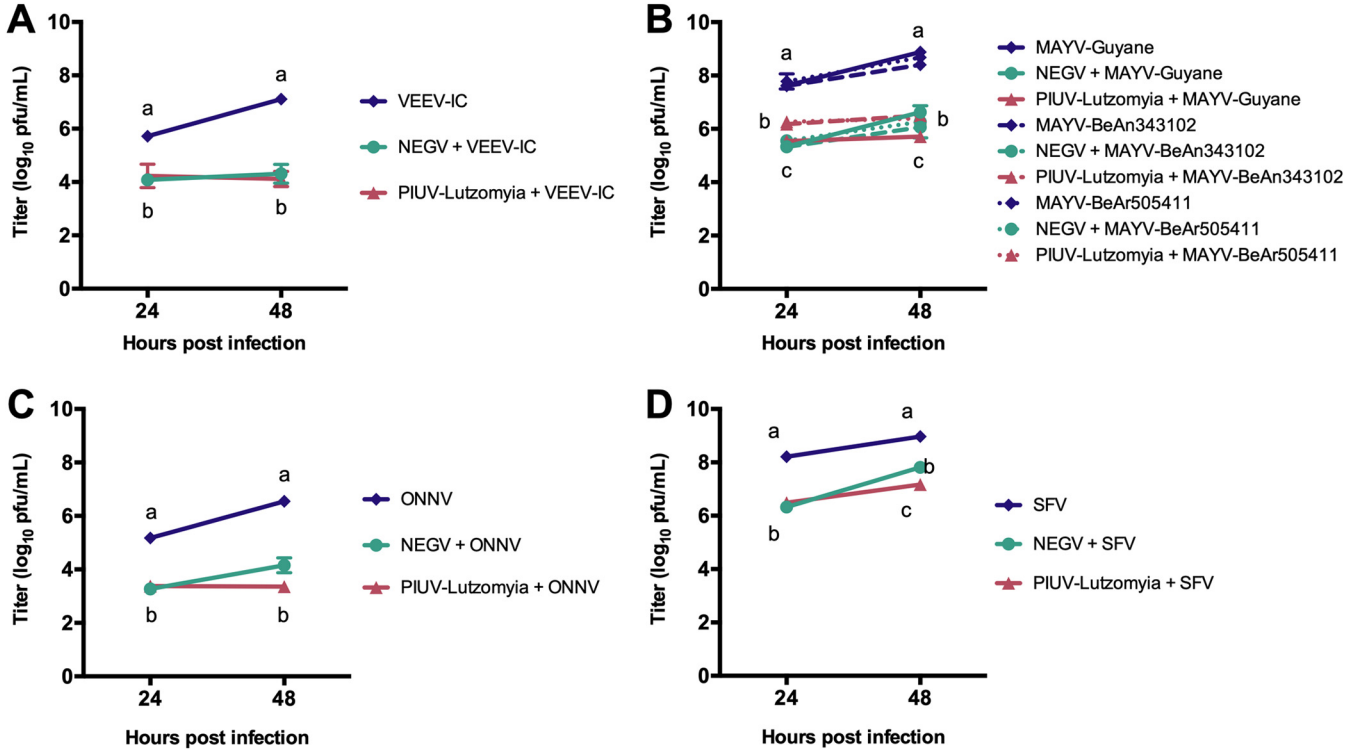

FIG 4 Growth curves of VEEV-IC, MAYV isolates, ONNV, and SFV in C7/10 cells during coinfections with wild-type negeviruses. (A) Growth curves of VEEV-IC when inoculated on cells at 0 h after negevirus infections. (B) Growth curves of MAYV-Guyane, MAYV-BeAn343102, and MAYV-BeAr505411 when inoculated on cells at 0 h after negevirus infection. (C) Growth curves of ONNV when inoculated on cells at 0 h after negevirus infection. (D) Growth curves of SFV when inoculated on cells at 0 h after negevirus infection. Negeviruses were inoculated at an MOI of 1 for all conditions. Alphaviruses were inoculated at an MOI of 0.1 for all conditions. All points represent means from $n = 3$, ±SD. Letters indicate significant differences ($P < 0.0001$).

isolates expressing GFP or wild-type NEGV only modestly reduced CHIKV titers. The use of parastransgenic NEGV expressing scFvs demonstrates a novel approach to disrupt pathogen infection in mosquitoes. This method adapts two existing techniques for pathogen control: *Wolbachia*-infected mosquitoes and the CRISPR-Cas-aided integration of scFv sequences into the mosquito genome. *Wolbachia* is a ubiquitous species of bacteria found in many insects that has been shown to block replication of some viral pathogens in cell cultures and mosquitoes. The use of *Wolbachia*-infected vectors has been widely adapted to curb mosquito-borne viral diseases, propelled by its natural ability to colonize mosquitoes (40). Negeviruses also possess this attribute, having been discovered in numerous mosquito species on 6 continents, along with sandflies and other diverse insect species (20–23, 25). Insertion of gene-editing scFv sequences into mosquito genomes has also been used to prevent *Plasmodium* and DENV infection (41, 42). By using CRISPR-Cas9 to insert an scFv targeting *Plasmodium*, infection was blocked in *Anopheles* mosquitoes, and gene drive ensured the production of the scFv

TABLE 1 Rescue and passage of NEGV infectious clones with GFP inserted at different sites of the genome[a]

Insertion site	GFP expression P0	GFP expression P1	CPE
ORF1-2A-GFP	+	+	+
GFP-ORF2	+	−	−
ORF2-GFP	+	−	−
ORF2-2A-GFP	+	−	−
GFP-ORF3	+	−	−
ORF3-GFP	+	+	+
ORF3-2A-GFP	+	+	+

[a]Transcribed RNA was electroporated for passage 0 (P0) stock and supernatant was collected to generate P1 stock. CPE, cytopathic effect; +, presence; −, absence.

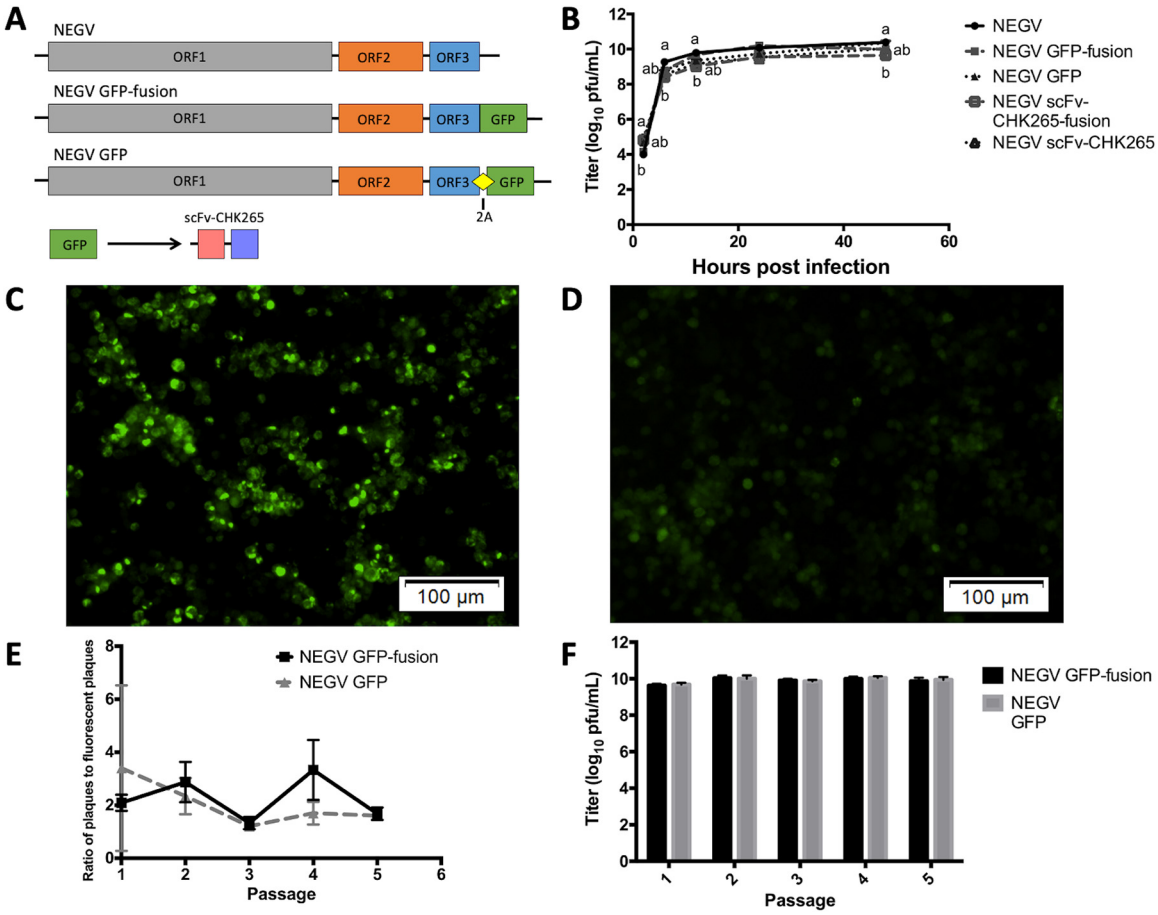

FIG 5 Rescued paratransgenic NEGV infectious clones. (A) Schematic of NEGV genomes for wild-type and GFP-expressing viruses. NEGV GFP-fusion added the GFP sequence onto ORF3, and NEGV GFP separated the ORF3 and GFP with a 2A sequence to produce the proteins separately. GFP was replaced by scFv-CHK265 for NEGV scFv-CHK265-fusion and NEGV scFv-CHK265. (B) Growth curves of NEGV wild type and NEGV mutants expressing GFP or scFv-CHK265. All points represent means from $n = 3$, ±SD. Letters indicate significant differences ($P < 0.0001$). (C) Fluorescent microscopy of C7/10 cells infected with NEGV GFP-fusion. Cells demonstrate brilliant, punctate fluorescence. (D) Fluorescent microscopy of C7/10 cells infected with NEGV GFP. Cells demonstrate dull, diffuse fluorescence. (E) The ratio of plaques to fluorescent plaques over 5 passages in C7/10 cells with NEGV GFP-fusion and NEGV GFP. All points represent means from $n = 3$, ±SD. (F) Titer of NEGV GFP-fusion and NEGV GFP during 5 passages in C7/10 cells. All points represent means from $n = 3$, ±SD. All NEGV isolates were inoculated at an MOI of 1.

in the offspring. In this study, we used an scFv expression strategy by cloning an anti-CHIKV scFv into the NEGV genome. Using NEGV as a vehicle for paratransgenesis is advantageous, because an isolate can infect multiple host species, and it is suspected to be vertically transmitted in mosquitoes and, in theory, could become established in multiple generations of the infected host species (17, 22, 43).

Modifications to certain parts of the NEGV genome were tolerated as both cleaved and fusion proteins. Expression of extraneous proteins in viruses is common with 2A sequences to produce separate proteins or under a separate subgenomic promoter (44, 45). However, extraneous proteins expressed as a fusion with a structural virus protein is uncommon. ORF3 is ~25 kDa and is suspected to be the membrane protein, the dominant structural protein, and ORF2 is ~40 kDa and is the putative glycoprotein predicted to form a bud projecting from one end of the virion (26). The viability of the NEGV isolates with GFP- or scFv-fusion at ORF3 isolates was surprising, because these inserts double the size of the membrane protein, which must interact with itself and ultimately support the projection of the glycoprotein. The modifications to ORF2 resulting in nonviable virus are not surprising, as the glycoprotein is suggested to be important for cell attachment and entry (26). GFP preceded by a 2A sequence was also

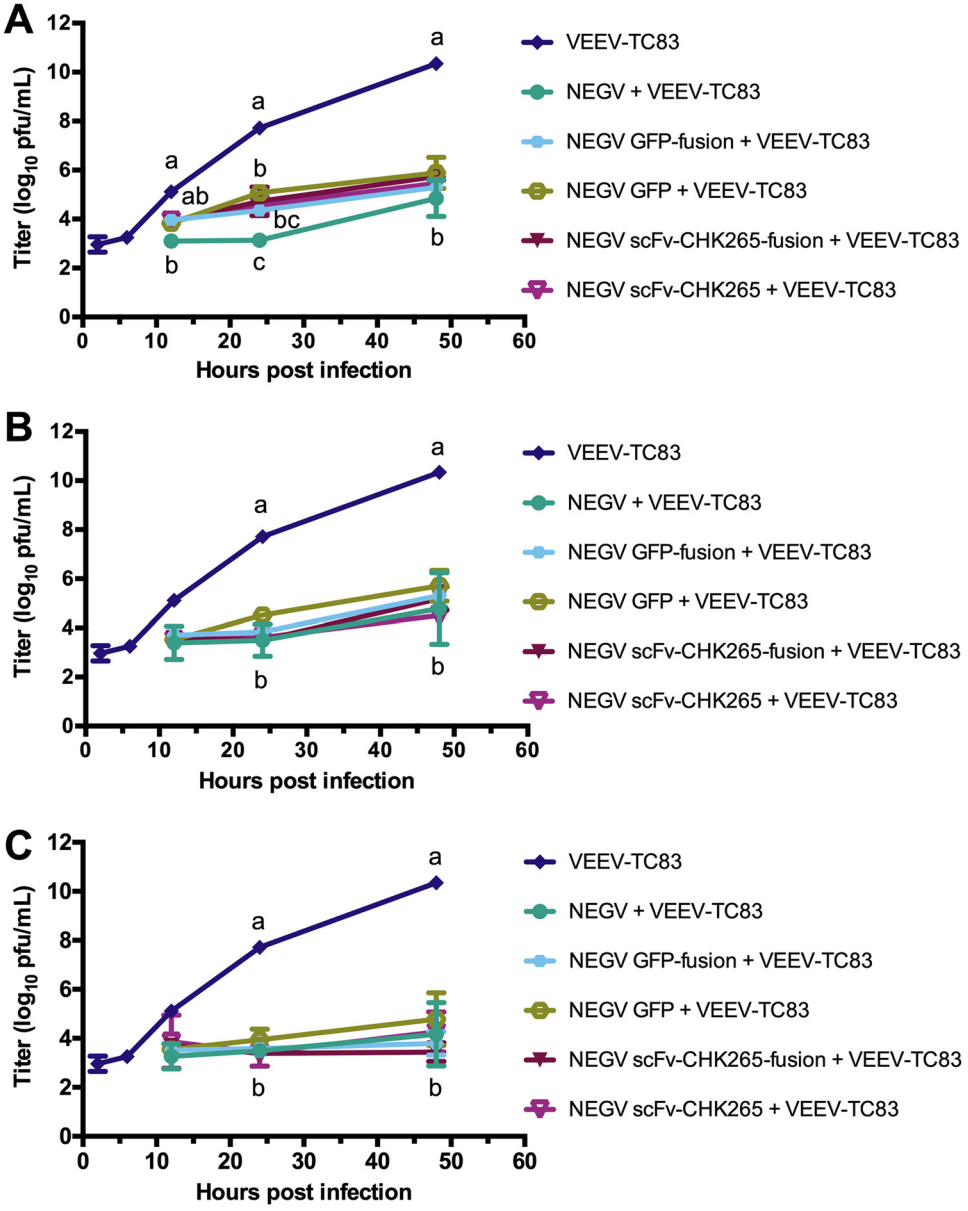

FIG 6 Growth curves of VEEV-TC83 during coinfections with paratransgenic NEGV. Growth curves of VEEV-TC83 when inoculated on cells at 0 h after NEGV infection (A), 2 h after NEGV infection (B), and 6 h after NEGV infection (C). All NEGV isolates were inoculated at an MOI of 1. VEEV-TC83 was inoculated at an MOI of 0.1 for all conditions. All points represent means from $n = 3$, ±SD. Letters indicate significant differences ($P < 0.0001$).

successfully cloned onto the C terminus of ORF1. As 2A allows for separation of the two proteins, this insertion only added 17 residues to an ~268-kDa protein. However, ORF1 will likely be expressed at lower levels than ORF3. By using NEGV to express anti-CHIKV scFvs, the cleaved and fused inserts may provide distinct advantages. Cleaved scFvs are free to be transported around the cell, accessing many different locations where they may encounter CHIKV proteins. In contrast, fused proteins are bound to the membrane protein of NEGV and are limited to compartments of the cell where NEGV proteins are expressed and virions are assembled. In theory, increasing the concentration of the scFvs in specific areas of the cell should inhibit CHIKV virion assembly and egress. By using both cleaved and fused NEGV isolates, the scFv sequence can also be easily replaced to target a new pathogen, adding to the versatility of this technique.

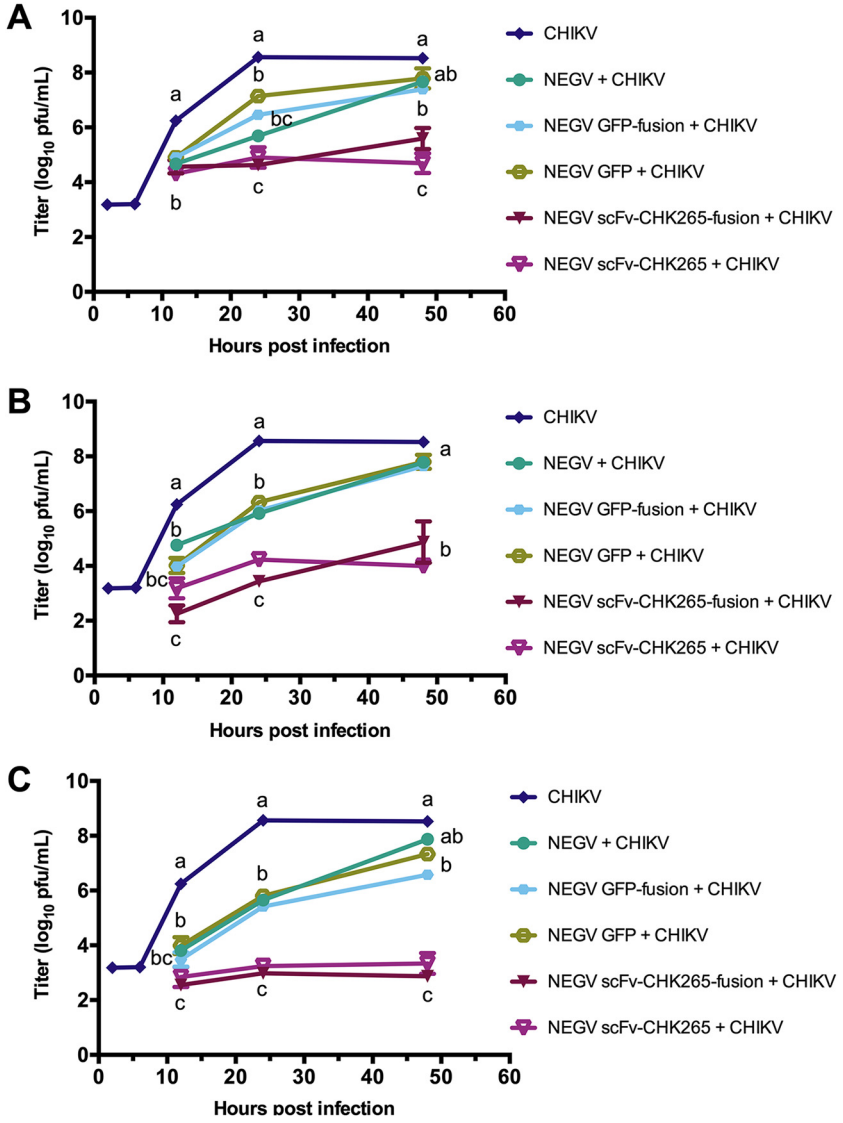

FIG 7 Growth curves of CHIKV during coinfections with paratransgenic NEGV. Growth curves of CHIKV when inoculated on cells at 0 h after NEGV infection (A), 2 h after NEGV infection (B), and 6 h after NEGV infection (C). All NEGV isolates were inoculated at an MOI of 1. CHIKV was inoculated at an MOI of 0.1 for all conditions. All points represent means from $n = 3$, ±SD. Letters indicate significant differences ($P < 0.0001$).

The current experiments demonstrate the ability of some negeviruses, both wild-type and paratransgenic isolates, to inhibit replication in mosquito cells with coinfected arboviruses. The next question is whether genetically altered negeviruses will survive and replicate in live mosquitoes and, if so, whether they will be vertically transmitted or transovarially transmitted in the insects. This will be our next area of investigation. If successful, then the use of paratransgenic negeviruses could be another novel method to alter the vector competence of mosquitoes for selected arboviruses.

MATERIALS AND METHODS

Cell culture and viruses. *Aedes albopictus* (C7/10) cells (46) were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). African green monkey kidney (Vero E6) cells were obtained from the American Type Culture Collection (ATCC). C7/10 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% minimal essential medium nonessential amino acids, 1% tryptose phosphate broth, and 0.05 mg/ml

gentamicin in a 30°C incubator with 5% CO_2. Vero cells were maintained in DMEM supplemented with 10% FBS and 0.05 mg/ml gentamicin in a 37°C incubator with 5% CO_2.

NEGV was rescued in C7/10 cells from an infectious clone, as previously described, without further passage (47). The sequence was derived from NEGV strain M30957, isolated from a pool of *Culex coronator* mosquitoes collected in Harris County, TX, in 2008 (17). Piura virus (PIUV) strain EVG 7-47 (PIUV-Culex) was isolated from a pool of *Culex nigripalpus* mosquitoes from Everglades National Park, FL, in 2013 (22). PIUV EVG 7-47 was passaged four times in C6/36 cells and obtained from the WRCEVA. PIUV strain CO R 10 (PIUV-Lutzomyia) was isolated from a pool of *Lutzomyia evansi* sandflies caught in Ovejas, Sucre, Colombia, in 2013 (22). The isolate PIUV CO R 10 was passaged twice in C6/36 cells and also obtained from the WRCEVA. CHIKV isolate 181/25 (48) was rescued in Vero cells from an infectious clone, as previously described (49). Rescued CHIKV 181/25 was subsequently passaged once in C7/10 cells and once in Vero cells. VEEV vaccine strain TC-83 (50) was rescued in baby hamster kidney (BHK) cells from an infectious clone without further passage. VEEV isolate P676 (VEEV-IC) and SFV isolate A774/C2/A were attained from Public Health England and passaged once in Vero cells. ONNV isolate UgMP30 and MAYV isolates Guyane, BeAn344102, and BeAr505411 were attained from BEI Resources and passaged once in Vero cells.

Cloning NEGV for exogenous gene expression. The NEGV infectious clone was used as the backbone to express exogenous genes. Green fluorescent protein (GFP; 717 bp) was inserted along several sites of the NEGV genome, and the scFv of anti-CHIKV neutralizing antibody CHK265 (771 bp, including linkers) (39) was inserted on the C terminus of ORF3 as either a fusion protein or with a 2A sequence (EGRGSLLTCGDVEENPGP) (Fig. 1A). The cloned scFv CHK265 sequence contained an N-terminal linker (LAAQPAMA) for articulation from the viral ORF3 protein and a domain linker [$(G_4S)_4$] between the variable heavy (V_H) and variable light (V_L) domains (Integrative DNA Technologies) (Fig. 1B). Cloning was performed using an In-Fusion HD cloning kit (TaKaRa Bio) per the manufacturer's protocol. Correct insertion was confirmed by sequencing. Infectious clones of NEGV containing exogenous genes were rescued in C7/10 cells as previously described and without further passage (47). Passaging of NEGV isolates expressing GFP was performed by inoculating C7/10 cells at an MOI of 1 and collecting medium supernatant at 48 h postinfection.

Virus growth curves. Negevirus and alphavirus growth curves were done in C7/10 cells maintained at 30°C and 5% CO_2. Negeviruses were inoculated at an MOI of 1 or 5. Alphaviruses were inoculated at an MOI of 0.1. Virus was added to the cells, which were incubated at 30°C for 1 h. Inoculum was removed, cells were washed with phosphate-buffered saline (PBS), and fresh medium was added to the wells. Cells were incubated in a 30°C incubator with 5% CO_2. Samples were collected in triplicate at 2, 6, 12, 24, and 48 h postinfection (hpi). Samples were clarified by centrifugation at 1,962 $\times$ *g* for 5 min. Supernatant was removed and stored at −80°C until used for plaque assays. Negevirus titers were determined by plaque assay in C7/10 cells as previously described (47). Alphavirus titers were determined by standard plaque assay in Vero cells.

Negevirus-alphavirus coinfections. C7/10 cells were inoculated with negevirus isolates at an MOI of 1 or 5 to establish infection in a high proportion of cells. The cells were also inoculated with an alphavirus at an MOI of 0.1 at 0, 2, or 6 h after negevirus infection. Medium was removed after 1 h of simultaneous incubation with negevirus and alphavirus inocula. Cells were then washed with PBS, and fresh medium was added to the wells. Cells were held in a 30°C incubator with 5% CO_2. Samples were collected in triplicate at 12, 24, and 48 h after alphavirus infection. Samples were clarified by centrifugation at 1,962 $\times$ *g* for 5 min. Supernatant was removed and stored at −80°C until used for plaque assays. Alphavirus titers were determined by standard plaque assay in Vero cells.

Statistical analysis. Differences in virus growth curves were determined by two-way analysis of variance, followed by Tukey's test. Comparison of NEGV growth curves with different MOIs was determined by multiple *t* tests, followed by the Holm-Sidak method. All statistical tests were performed using GraphPad Prism 6.0.

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E.I.P. designed the experiments. E.I.P., T.K., M.A.C.-G., and H.G. completed the experiments. E.I.P. undertook analysis. E.I.P., T.K., R.B.T., G.L.H., and N.L.F. wrote and edited the manuscript, and all authors agreed to the final version. R.B.T., G.L.H., and N.L.F. provided supervision. E.I.P. and N.L.F. acquired the funding.

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